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Peptidome profiling of venom from the social wasp *Polybia paulista*

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ABSTRACT

Most crude venom from *Polybia paulista* is composed of short, linear peptides; however, only five of these peptides are structurally and functionally characterized. Therefore, the peptides in this venom were profiled using an HPLC-IT-TOF/MS and MS^n system. The presence of type *-d* and *-w* ions that are generated from the fragmentation of the side chains was used to resolve I/L ambiguity. The distinction between K and Q residues was achieved through esterification of the α- and ε-amino groups in the peptide chains, followed by mass spectrometry analysis. Fourteen major peptides were detected in *P. paulista* venom and sequenced; all the peptides were synthesized on solid-phase and submitted to a series of bioassays. Five of them had been previously characterized, and nine were novel toxins. The novel peptides correspond to two wasp kinins, two chemotactic components, three mastoparans, and two peptides of unknown function. The seven novel peptides with identified functions appear to act synergistically with the previously known ones, constituting three well-known families of peptide toxins (wasp kinins, chemotactic peptides, and mastoparans) in the venom of social wasps. These multifunctional toxins can cause pain, oedema formation, haemolysis, chemotaxis of PMNLs, and mast cell degranulation in victims who are stung by wasps.

1. Introduction

The venoms of wasps, bees and ants are generally used both for self-defence and to repel predators or intruders from their nest (Palma, 2013). The stings of these insects cause severe pain, local oedema and erythema caused by an increase in the permeability of the blood vessels near the skin, local tissue damage and occasionally death in large vertebrates, including humans (Brigatte et al., 2011). Wasp venoms are composed of low molecular weight compounds, proteins and peptides (Nakajima, 1984), with peptide components constituting approximately 70% of the venom toxins (De Souza and Palma, 2009). Different types of inflammatory peptides have been reported in these venoms, including mastoparans (De Graaf et al., 2009), protonectin-like toxins, chemotactic peptides and wasp kinins (De Souza et al., 2005).

These peptides contain pharmacological actions including mast cell degranulation, chemotaxis of polymorphonuclear leukocytes (PMNL), cytolysis and smooth muscle contraction (Mendes and Palma, 2006; Palma, 2011). The identification of the peptides in these venoms is important for characterizing the pharmacological symptoms observed during the envenoming process. This knowledge will to help physicians assist the sting victims. The pharmacological properties of wasp venoms have not been fully investigated because of the limited availability of wasp venom and the low abundance of each individual peptide in these venoms.

Venom profiling is a basic approach to global venom exploration by mass spectrometry. The data set generated by this profiling, with or without chromatographic separation, produces a global picture of the venom and reveals the complex composition of venom (Escoubas et al., 2008a, b). Until a few years ago, studies to elucidate the molecular complexity of venoms were based on low-resolution chromatographic methods, which were able to detect only the most abundant toxins (Escoubas et al., 2008a, b). Traditional fractionation usually requires the use of large-scale biological material with manual fraction collection and focuses on only the most abundant toxins while ignoring the minor components. The combination of high-resolution LC systems with modern mass spectrometers is making the determination of venom compositions more comprehensive and more accurate. Combination techniques, such as LC-MS, are powerful analytical tools for detecting and sequencing

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tiny amounts of peptides, even in complex mixtures (Jia et al., 2006).

Proteomic and peptidomic studies generally use LC-ESI-MS and nano-ESI-MS/MS protocols that enable the rapid and sensitive identification and characterization of proteins/peptides. These experimental approaches have led to the identification of approximately 100 different peptide components in snake venoms (Jia et al., 2006). Similar approaches for venom from scorpions, spiders and marine snails of the genus *Conus* has resulted in the identification of hundreds of different peptide toxins (Calvette et al., 2007; Conlon et al., 2006; Dyason et al., 2002; Escoubas, 1998; Escoubas et al., 2008a, b; Newton et al., 2007; Liang, 2008; Mandal et al., 2007; Bias et al., 2009; Davis et al., 2009). The chemical diversity of peptides from the venoms of social wasps is still not well understood. The venom from *Agelaia pallipes pallipes* contains nine identified active peptides (Baptista-Saidemberg et al., 2011). Meanwhile, the venom of *Polybia paulista* was characterized by mass spectrometry-based strategies as containing dozens of short peptides (Dias et al., 2014). However, only a few peptide components have had sequences assigned for these wasp species (De Souza et al., 2005; Mendes and Palma, 2006; Baptista-Saidemberg et al., 2011; Rocha et al., 2010) In light of the medical consequences of social wasp stings in Neotropical regions of the planet and the complex peptide composition of their venoms, we used peptides fractionation with reversed-phase chromatography coupled to electrospray ionization-ion trap-time of flight mass spectrometry (LC-ESI-IT-TOF/MS) and MS^n analysis to profile the peptidome of the venom from *P. paulista*, which is one of the most aggressive species of social wasps from Brazil and Central America.

2. Materials and methods

2.1. Biological material

The social wasp *Polybia paulista* was collected in Rio Claro-SP, southeast Brazil (S22°23′51.1″; W047°32′54″) and immediately frozen and stored at −20 °C and transported to the laboratory. After unfreezing the wasps their venom reservoirs of were removed by dissection with surgical microscissors and washed with 50% (v/v) acetonitrile (MeCN, Aldrich) containing 0.1% (v/v) trifluoroacetic acid (TFA, Aldrich) and cocktail of protease inhibitors (2 mM AEBSF, 0.3 μM Aprotinin, 130 μM Bestatin, 1 mM EDTA, 14 μM E-64 and 1 μM Leupeptin) (Sigma–Aldrich, Saint Louis, USA) to solubilize the peptides. The extract was centrifuged at 10,000 × g for 20 min at 4 °C. The resulting pellets were discarded and the supernatant was lyophilized and stored at −85 °C. This extract corresponds to the peptide-rich fraction.

2.2. Animals

Male Swiss mice weighing between 25 and 30 g were used throughout this study. Mice were housed under controlled humidity at 22 °C ± 1 and subjected to a 12 h light–dark cycle in a sound-attenuated room. Food and water were available *ad libitum*, and mice were taken to the testing room at least 1 day before the experiment. All behavioural testing was performed between 9:00 am and 4:00 pm. Each mouse was used only once. All experiments were performed in accordance with the guidelines for the ethical use of conscious animals in pain research, published by the National Academy of Sciences (http://www.nap.edu/catalog/5140.html). The procedures were approved by the Institutional Animal Care Committee at São Paulo State University, UNESP campus Rio Claro, SP (CEUA-IB-UNESP-CRC, Protocol nº 1984). Efforts were made to minimize the number of animals used and their suffering.

2.3. Venom fractionation

About 45 μg of peptide-rich fraction from *P. paulista* venom (corresponding to the material from 10 individuals) was solubilized in 200 μL 50% (v/v) MeCN and fractionated on an RP-HPLC system using an X-Bridge BEH 130 C-18 column (100 mm × 2.1 mm; 3.5 μm) (Waters, Massachussetts, USA) at a flow rate of 200 μL/min. The elution was initially performed under isocratic conditions for 5.0 min with 5% (v/v) MeCN (containing 0.1% (v/v) TFA) followed by a linear gradient from 5 to 60% (v/v) MeCN (containing 0.1% (v/v) TFA) between 5.1 and 42.0 min at 28 °C. The eluent was monitored at 215 nm with a UV-DAD detector, mod. SPD-M10A (Shimadzu, Kyoto, Japan). The fractions were manually collected in 5-mL glass vials. The homogeneity of each peak was determined by ESI-MS analysis.

2.4. Mass spectrometric analysis

MS and MS^n analyses were conducted on an ion trap/time-of-flight mass spectrometer (IT-TOF/MS) (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source. The instrument was set to permit the accumulation of all ions in the octapole, followed by rapid pulsing into the IT for MS^n analysis. Then, the sample entered the TOF sector for accurate mass determinations. The setting conditions for optimized operations were as follows: positive mode, electrospray voltage 4.5 kV, CDL temperature 200 °C, block heater temperature 200 °C, nebulizer gas (N_2) flow of 1.5 L/min, trap cooling gas (Ar) flow of 95 mL/min, ion trap pressure 1.7×10^{-2} Pa, TOF region pressure 1.5×10^{-4} Pa, ion accumulation time 50 ms, collision energy set at 35% both for MS_2 and MS_3, and collision gas set to 20%. Instrument calibration was performed using auto-tuning, carried-out in presence of Na-TFA solution (TFA 0.1% (v/v) 10 mM NaOH at pH 3.5). During auto tuning process the control parameters for the MS instrument are automatically optimized in accordance with a default protocol defined by the instrument producer. After this routine calibration mass spectral resolution was around 10,000 FWHM, and error around 3.08 ppm.

2.5. Peptide synthesis

The peptides were synthesized by step-wise manual solid phase synthesis using N-9-fluorophenylmethoxy-carbonyl (Fmoc) chemistry with Novasyn TGS resin (NovaBiochem, Germany). Side-chain protective groups included t-butyl for serine and t-butoxycarbonyl for lysine. Cleavage of the peptide–resin complexes was performed by treatment with a mixture of trifluoroacetic acid, 1,2-ethanedithiol, anisole, phenol, and water (82.5:2.5:5:5:5 by volume, respectively). The cleavage reaction used 10 mL of mixture per gram of complex and was incubated at room temperature for 2 h. Samples were filtered to remove the resin, and ethyl ether was added at 4 °C to precipitate the crude peptides. The samples were centrifuged at 1000 g for 15 min at room temperature, and the supernatant was discarded. The crude peptide pellets were solubilized in water and separated on an RP-HPLC system using a semi-preparative column (SHISEIDO C18, 250 mm × 10 mm, 5 μm) under isocratic conditions at a flow rate of 2 mL/min. The elution was monitored at 215 nm with a UV-DAD detector, mod. SPD-M10A (SHIMADZU, Kyoto, Japan), and each fraction eluted was manually collected in 2 mL plastic vials. The homogeneity and correct sequence of the synthetic peptides were evaluated by comparing their retention times during RP-HPLC under isocratic conditions, as reported above for the natural peptides. ESI-MS analysis was also used to check the purity of peptides (given the presence of a single molecular ion, equivalent to the expected molecular mass for the amino sequence of each peptide).

2.6. Reduction and carbamidomethylation of cysteine residues

Peptides (1 μg/μL) were solubilized in 100 μL of 25 mM ammonium bicarbonate buffer at pH 8.5. A solution of 50 mM dithiothreitol (DTT) (Amersham Biosciences, Sweden) was prepared in ammonium bicarbonate buffer solution as described above. An aliquot of 25 μL of DTT solution was added to the sample. The resulting solution was homogenized, incubated at 40 °C for 30 min and then cooled to room temperature. Then, 25 μL of 100 mM iodoacetamide (IAA) (Amersham Biosciences, Sweden) in the ammonium bicarbonate buffer solution was added to the mixture. The resulting solution was left to react for 60 min in the dark at room temperature.

2.7. Acetylation of lysine residues

The acetylation of Lys residues was used to distinguish between the isobaric amino acid residues of Lys and Gln. The peptides were submitted to acetylation as follows. The acetylation reagent was prepared by mixing 20 μL acetic anhydride with 60 μL methanol, and the peptide of interest (1 nmol) was solubilized in 20 μL of 50 mM ammonium bicarbonate pH 7.0. Then, 50 μL of the acetylation reagent was mixed with 20 μL of the peptide solution and incubated at room temperature (~25 °C) for 60 min. Next, the mixture was lyophilized to dryness and reconstituted in 50% acetonitrile for analysis by mass spectrometry.

2.8. Biological assays

2.8.1. Haemolytic activity

Washed rat red blood cells (WRRBC) were used to evaluate the haemolytic activity of the peptides. WRRBC were prepared by washing 50 mL of Wistar RRBC suspension three times with physiological saline solution (0.85% (w/v) NaCl and 10 mM $CaCl_2$) and re-suspending in 50 mL of the same solution. Aliquots of WRRBC (0.5% v/v) were then incubated at 37 °C in the presence of each synthetic peptide for 120 min with gentle mixing. Samples were then centrifuged and the absorbance of the supernatants was measured at 540 nm. The absorbance measured from WRRBC lysed in the presence of 1% (v/v) Triton X-100 was considered 100%.

2.8.2. Mast cell degranulation activity

Mast cell degranulation was determined by measuring the release of β-D-glucosaminidase (co-localized with histamine) in the presence of the synthetic peptides as modified by De Souza et al. (2005). Mast cells were obtained by peritoneal washing of adult Wistar rats with a solution containing 0.877 g NaCl, 0.028 g KCl, 0.043 g NaH_2PO_4, 0.048 g KH_2PO_4, 0.10 g glucose, 0.10 g BSA, 90 mL of a 2 M $CaCl_2$ solution and 50 μL Liquemine (heparin, ROCHE) in 100 mL water. Mast cells were incubated in the presence of peptides for 15 min at 37 °C. After centrifugation, the supernatants were sampled by a β-D-glucosaminidase assay. Briefly, 50 μL of mast cell suspension was added to 50 μL of the substrate [3 mg of p-nitrophenyl-N-acetyl-β-D-glucosaminidine (Sigma–Aldrich, Saint Louis, USA) dissolved in 10 μL of 200 mM sodium citrate at pH 4.5] and incubated for 6 h at 37 °C. The reaction was stopped by the addition of 150 μL of 0.2 M Tris at pH 9.0 and the absorbance of coloured product was measured at 405 nm in a microtiter plate reader (Biotrack, Amersham Bioscience, Sweden). The values are expressed as the percentage of total β-D-glucosaminidase from lysed mast cells in the presence of 0.1% (v/v) Triton X-100.

2.8.3. Chemotaxis activity

Chemotaxis was assayed in a specific multi-chamber apparatus (Neuro Probe, USA) using polymorphonucleated leukocytes (PMNL) obtained from a subcutaneous inflammatory induction in Wistar rats. A special membrane with 10-μM pores was introduced between two chemotaxis apparatus parts that were open for leucocytes migration. The total cell concentration was 10^5 cells/mL. The upper chambers were filled with 200 μL of PMNL suspension (2.7×10^5 cells/mL in 0.9% (v/v) NaCl solution), and the lower chambers were filled with 400 μL of physiological solution containing the synthetic peptides. A polycarbonate membrane (10 μm pore diameter, Neuro Probe) was placed between the two chambers. The chemotaxis chamber was incubated at 37 °C for 1 h. After incubation, cells in the lower chambers were counted using a Neübauer chamber. The results are expressed as the number of migrating cells/mL and transformed in percentages of activity with respect to the standard peptide HR II peptide used as a positive control.

2.8.4. Von Frey electronic pressure-meter paw tests for mice

Hyperalgesia was induced by intraplantar (i.pl.) injection of carrageenan (300 μg) or peptides (10 and 30 μg/50 μL) into one of the hind paws. Mice were placed in acrylic cages (12 × 10 × 17 cm high) with a wire grid floor for 15–30 min before testing. During this adaptation period, the paws were poked 2–3 times. Before paw stimulation, the animals were quiet, without exploratory movements or defecation, and were not resting on their paws. In these experiments, we used a pressure-meter that consisted of a hand-held force transducer fitted with a 0.5 mm^2 polypropylene tip (electronic von Frey anaesthesiometer, IITC Inc., Life Science Instruments, Woodland Hills, CA, USA). The investigator applied the polypropylene tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hind paw. The test consisted of poking a hind paw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. In the electronic pressure-meter test, the intensity of the stimulus was automatically recorded when the paw was withdrawn. The maximal force applied was 18 g. The stimulation of the paw was repeated until the animal presented two similar measurements. If the results were inconsistent (i.e., a great difference in the baseline response compared to the other animals of the experiment), another animal was used. The results are reported as the Δ (delta) withdrawal threshold (g), calculated by subtracting the values obtained in absence of the peptides, from those obtained in presence of each peptide individually. The results are expressed as the percentage of activity in relation to the positive control (carrageenan).

2.8.5. Evaluation of oedema

Oedema was induced by i.p.l. injection of carrageenan (300 μg) or secapin-2 (1, 2, 4, 10, 30 and 50 μg) into one of the hind paws. The volume increase (oedema) in each injected paw up to the tibiotarsal articulation was measured using a digital paquimeter (Mitutoyo, CD-6″ CSX-B model, Brazil). The difference between the values obtained for both hind paws is expressed as the per cent increase in paw volume and was used as a measure of oedema.

For evaluation of the nociceptive activity of the peptides, they were dissolved in sterile saline (0.005, 0.35, 1, 2, 10 and 30 μg) and administered by i.pl. injection into one hind paw. A hypodermic 26-G needle was inserted into the skin of the second hind footpad (to avoid back flow) and the tip of the needle was inserted into the central area of the hind paw in the same area where filaments and the tip of the pressure-meter were applied. The nociceptive activity was evaluated at different times (0-before treatments, 15, 30, 60, 120, 180, 240, 360, 480 and 1440 min) after treatment and compared to the control. Carrageenan (Marine Colloids, 300 μg) was diluted in sterile saline and was used as positive control; sterile saline was used as a negative control.

2.8.6. Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare the groups and doses over time. The factors analysed were time, treatments and the time vs. treatment interaction. When a significant time vs. treatment interaction was detected, a one-way ANOVA was followed by the Tukey test for each time point to distinguish dosage effects. One-way ANOVA followed by Tukey test was also used for dose–response curves for a single time point. Results with $P < 0.05$ were considered to be significant.

3. Results

The fractionation of *P. paulista* venom revealed 24 peaks, designated 1 to 24 (Fig. 1). Fractions 1 to 4 correspond to a series of low molecular mass compounds. Fractions 1 and 4 correspond to histamine and serotonin, respectively (identified by comparison against standard compounds). The components of fractions 2 and 3 were not identified. Meanwhile, fractions 6 to 19 correspond to peptides representing different types of inflammatory activities and were the focus of this study. The complete amino acid sequences of these peptides were assigned using ESI-IT-TOF-MS and MS^n analysis. The interpretation of the mass spectra obtained under collisional induced decomposition (CID) conditions was accomplished by subtracting the *m*/*z* values between consecutive *b*- or *y*-ions, which enabled the assignment of the sequences of fourteen peptides. This procedure permitted almost complete sequence assignments, with a few remaining ambiguities with relation to the isobaric residues I/L and K/Q. The I/L ambiguities for the residues positioned inside the peptide chain were solved using ion-fragments of *d*- and/or *w*-type, specific for the fragmentation of side chain of the I and L residues. The remaining I/L ambiguities in the C- and N-terminal residues were solved by synthesizing four sequences for each peptide (with I or L positioned at each termini), which in turn were submitted to chromatographic analysis (in the same conditions described above). The retention times of synthetic peptides were compared to the natural one. To address the K/Q ambiguity, the samples were derivatized with acetic anhydride and submitted to mass spectrometric analysis under CID conditions. The ε-amino group from the side chain of K residues and the α-amino group of the N-terminal residue of each peptide become acetylated, contributing in increments of 42 mass units per acetyl group that was incorporated in the peptide chain. The side chain of Q residue does not react with acetic anhydride.

For brevity, we are showing only the MS^2 spectra of the peptide components of each fraction studied without any derivatization while still showing the I/L and K/Q ambiguities (Fig. 2–5). Under the chromatographic conditions used in this study, the peptide components began to elute at 19.8 min (fraction 5). The deconvolution of the MS^2 spectrum of this fraction revealed a peptide component with a molecular mass of 845.42 Da (Fig. S1A in the supplemental information). The precursor ion with an m/*z* value 423.71 as $[M + 2H]^{2+}$ was selected for fragmentation under CID conditions (Fig. 2A). Analysis of this spectrum revealed the peptide sequence to be TGDSPDVR. The molecular mass of the natural peptide (845.40 Da) is consistent with the C-terminal residue in the acidic form. Therefore, the complete sequence of this peptide is TGDSPDVR-OH (Table 1).

In the time between 21.0 and 22.5 min, two overlapping peptide components eluted (fractions 6A and 6B) with molecular masses of 1073.58 Da (fraction 6A) and 1300.74 Da (fraction 6B) (Fig. S1B and S1C in the supplemental information). The complete sequencing interpretation of these peptides revealed: fraction 6A- RPPGFTPFR-OH, and fraction 6B – RARPPGFTPFR-OH (Table 1). At a retention time of 24.0 min, the eluted fraction 7 (Fig. 1) contained a molecular mass of 1516.79 Da (Fig.S1D in the supplemental information). The complete sequence assigned for this peptide was RARPPGFTPFRDT-OH (Table 1).

Fraction 8 eluted at 27 min with a molecular mass of 628.40 Da (Fig.S2A of the supplement information); the complete interpretation of this spectrum with the isobaric ambiguities revealed the sequence ILGTIL-NH2 (Table 1). Fraction 9 eluted at a retention time of 28.2 min with a molecular mass of 1051.70 Da (Fig. S2B in the supplemental information); the unambiguous sequence assignment of this peptide component revealed its complete primary

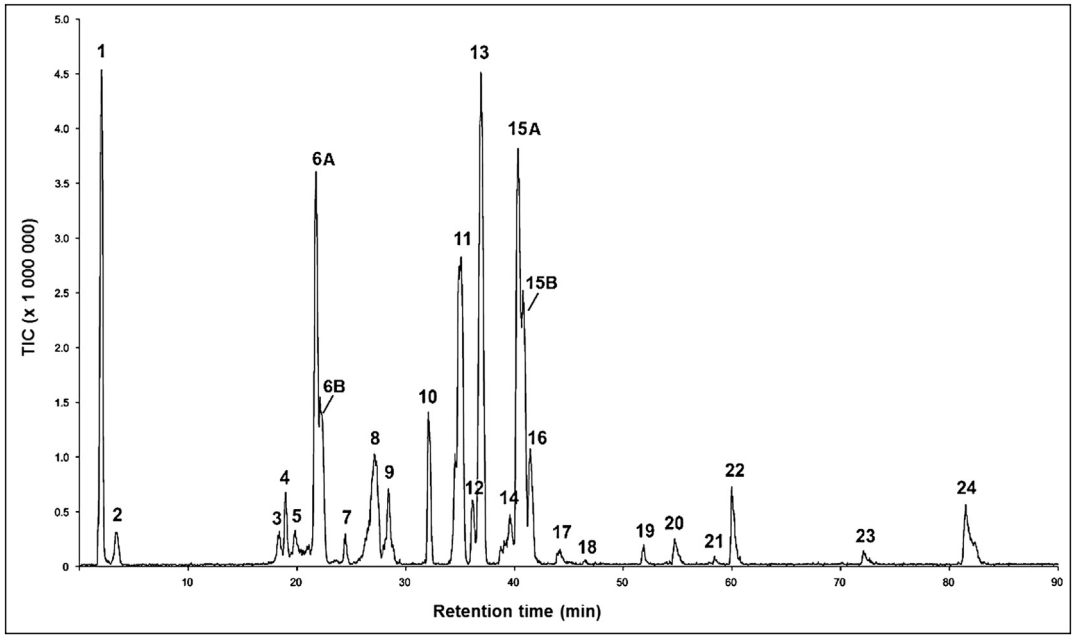

Fig. 1. Chromatographic profile for the crude venom of *P. paulista* by RP-HPLC with a packed X-Bridge BEH 130 C-18 (Waters) column (100 mm × 2.1 mm; 3.5 μm). The elution was performed under isocratic conditions from 0 to 5.0 min with 5% (v/v) MeCN (containing 0.1% (v/v) TFA), followed by a linear gradient from 5 to 60% (v/v) MeCN (containing 0.1% (v/v) TFA) in the interval from 5.1 to 90.0 min. The elution was carried out at 28 °C at a flow rate of 200 μL/min and monitored at 215 nm.

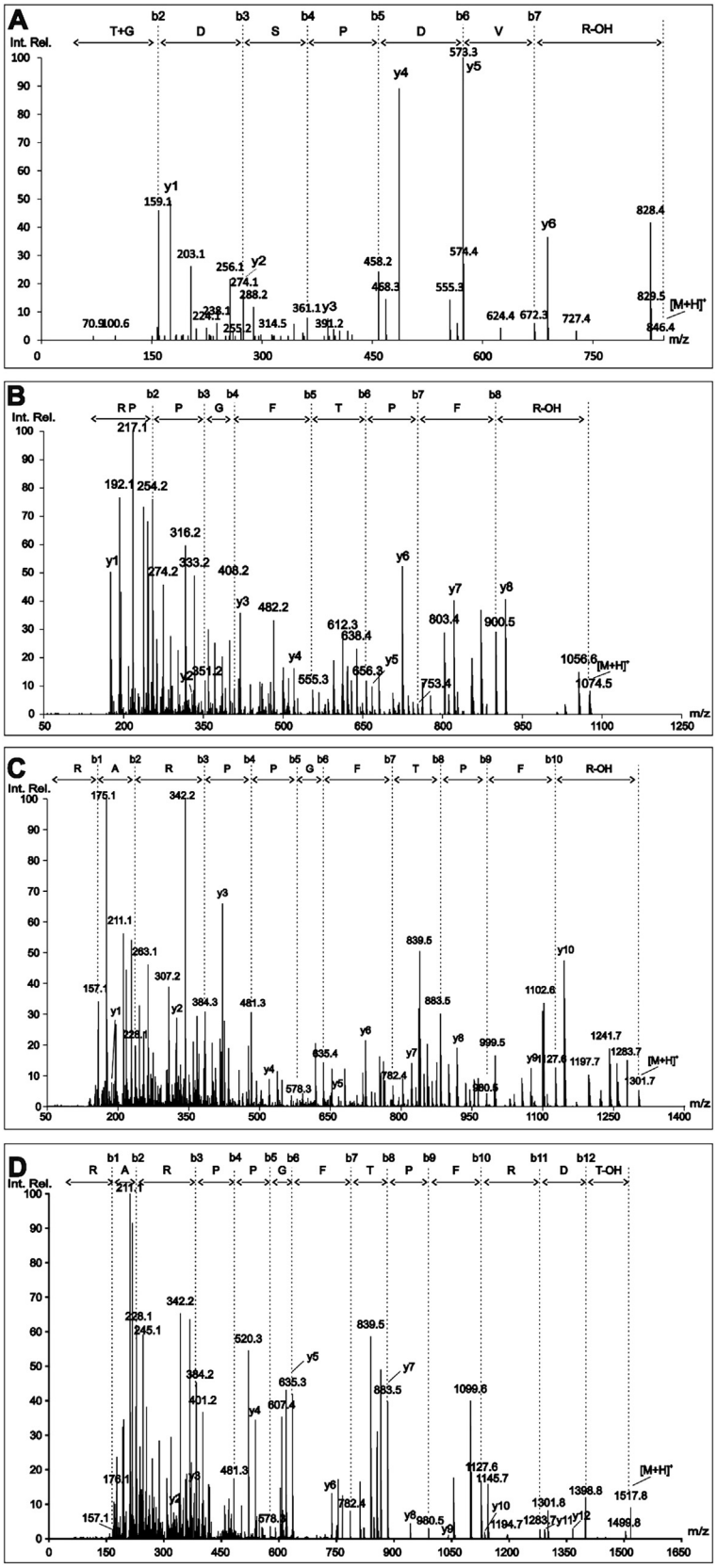

Fig. 2. MS^2 spectra obtained under CID conditions of fractions 5, 6A, 6B, and 7, in the positive mode, with the assignment of a series of *b*- and *y*-ions and de-convoluted to interpret and determine the peptide sequence. Some *d*- and *w*-ions that were used diagnostically to eliminate the ambiguity between the isobaric residues I/L are also assigned. The precursor-ions of *m*/*z* 423.71 as $[M + 2H]^{2+}$, *m*/*z* 537.78 as $[M + 2H]^{2+}$, *m*/*z* 651.37 as $[M + 2H]^{2+}$, and *m*/*z* 506.59 as $[M + 3H]^{3+}$ were selected for fragmentation in fractions 5 (**A**), 6A (**B**), 6B (**C**), and 7 (**D**), respectively.

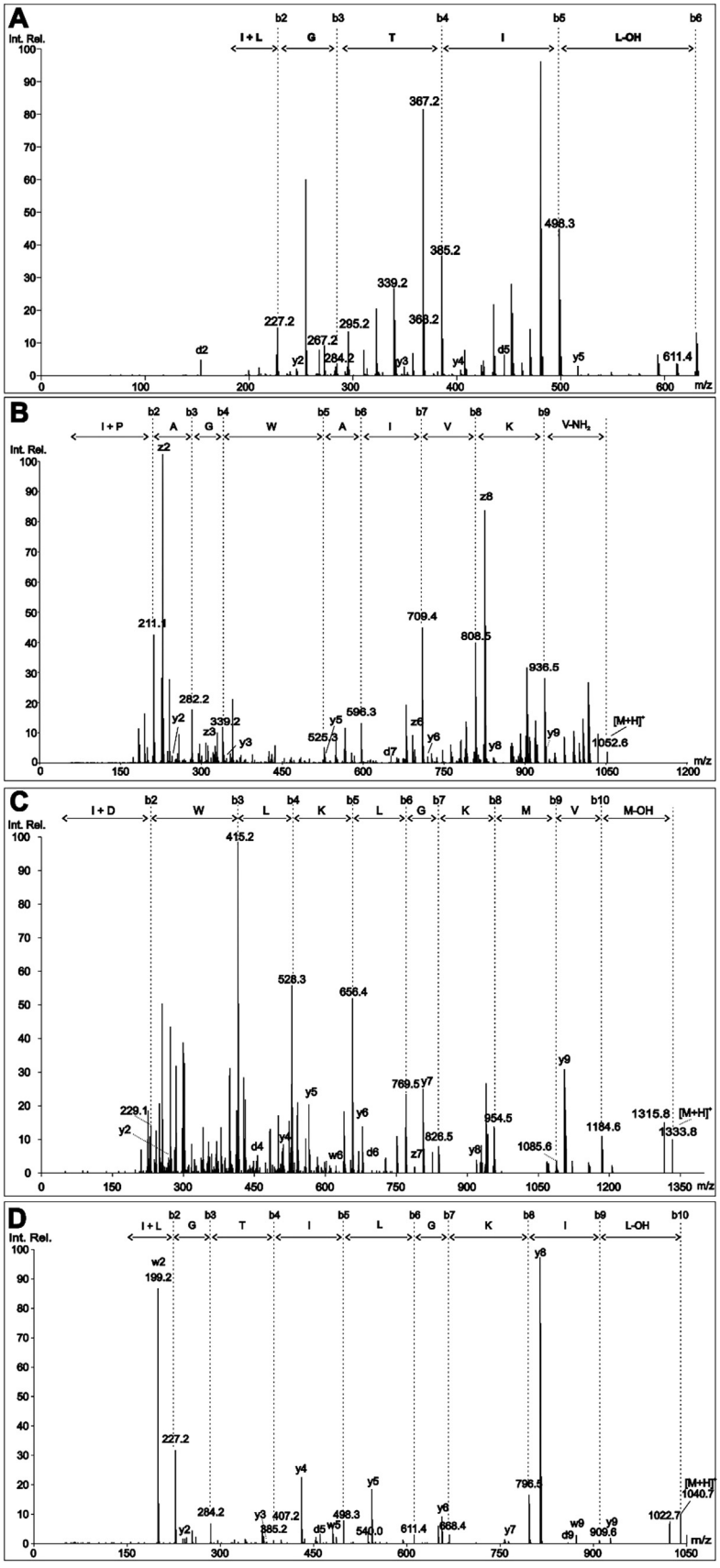

Fig. 3. MS^2 spectra obtained under CID conditions of fractions 8, 9, 10, and 11, in the positive mode, with the assignment of a series of *b*- and *y*-ions and de-convoluted to interpret and determine the peptide sequence. Some *d*- and *w*-ions that were used diagnostically to eliminate the ambiguity between the isobaric residues I/L are also assigned. The precursor-ions of *m*/*z* 629.4 as $[M + H]^+$, *m*/*z* 526.85 as $[M + 2H]^{2+}$, *m*/*z* 667.39 as $[M + 2H]^{2+}$, and *m*/*z* 520.86 as $[M + 2H]^{2+}$ were selected for fragmentation in fractions 8 (**A**), 9 (**B**), 10 (**C**), and 11 (**D**), respectively.

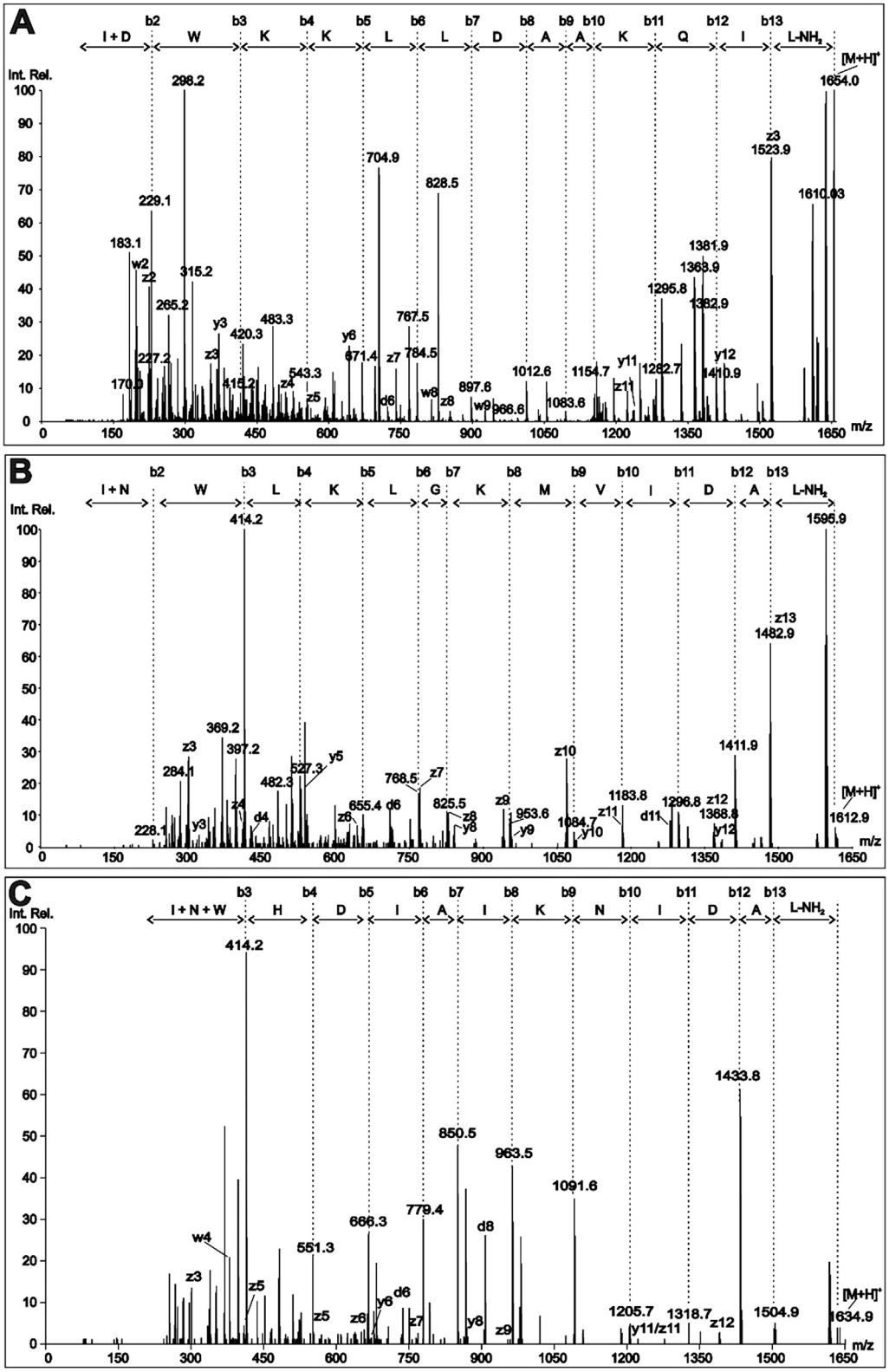

Fig. 4. MS^2 spectra obtained under CID conditions of fractions 13, 15A, and 15B, in the positive mode, with the assignment of a series of *b*- and *y*-ions and de-convoluted to interpret and determine the peptide sequence. Some *d*- and *w*-ions that were used diagnostically to eliminate the ambiguity between the isobaric residues I/L are also assigned. The precursor-ions of *m*/*z* 827.51 as $[M + 2H]^{2+}$, *m*/*z* 806.99 as $[M + 2H]^{2+}$, and *m*/*z* 817.97 as $[M + 2H]^{2+}$ were selected for fragmentation in fractions 13 (**A**), 15A (**B**), and 15B (**C**), respectively.

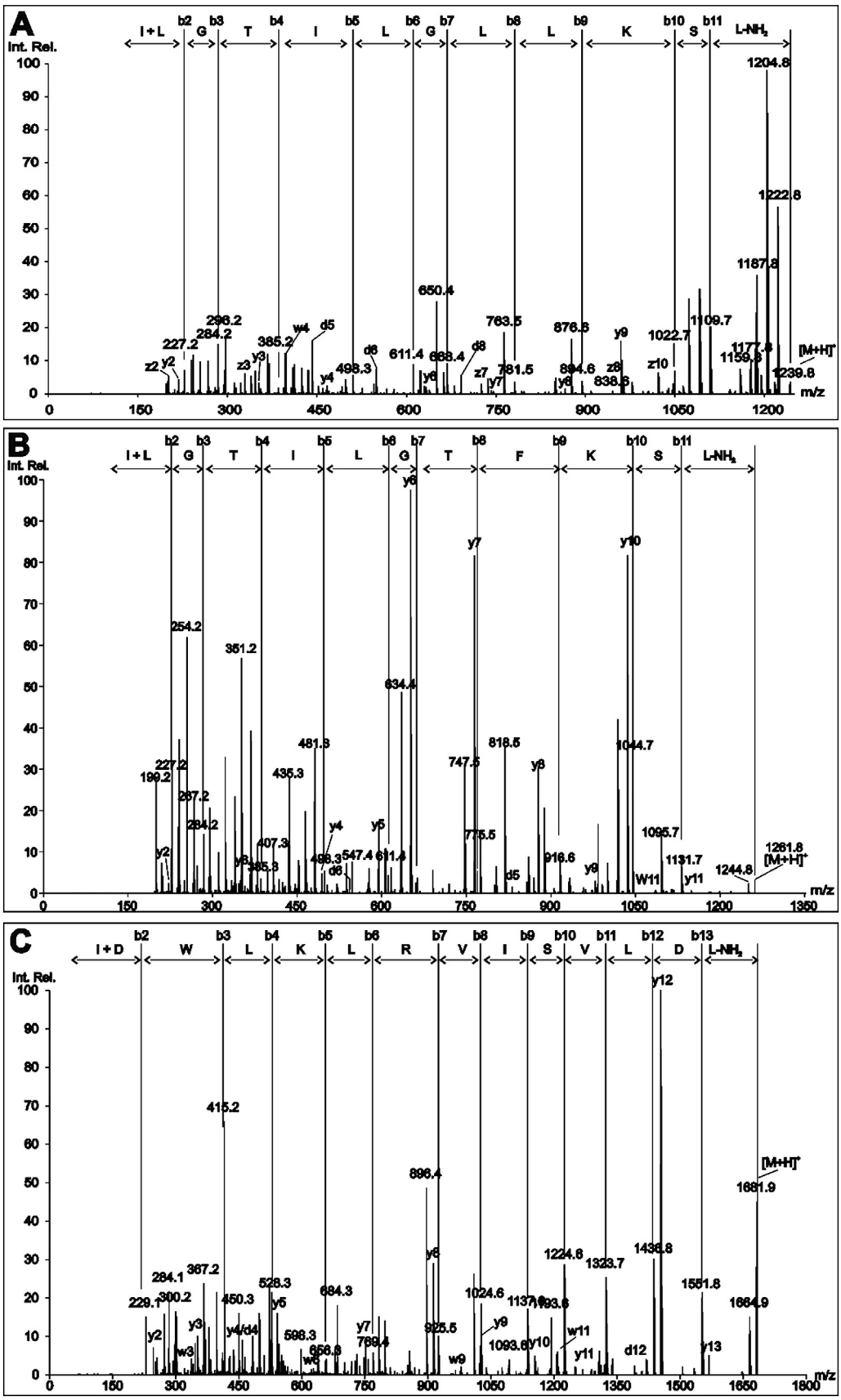

Fig. 5. MS^2 spectra obtained under CID conditions of fractions 16, 17, and 19, in the positive mode, with the assignment of a series of *b*- and *y*-ions and de-convoluted to interpret and determine the peptide sequence. Some *d*- and *w*-ions that were used diagnostically to eliminate the ambiguity between the isobaric residues I/L are also assigned. The precursor-ions of *m*/*z* 620.41 as $[M + 2H]^{2+}$, *m*/*z* 631.40 as $[M + 2H]^{2+}$, and *m*/*z* 841.47 as $[M + 2H]^{2+}$, were selected for fragmentation in fractions 16 (**A**), 17 (**B**), and 19 (**C**), respectively.

Table 1
Profile of peptides sequenced from the venom of the social wasp *P. paulista*.

Peptides	Sequences	Fractions	Comments
Wasp kinins			
Thr^6-Bradykinin	R P P G F T P F R–OH	6A	Picolo et al., 2010
RA-Thr^6-Bradykinin	R A R P P G F T P F R–OH	6B	Novel peptide
RA-Thr^6-Bradykinin-DT	R A R P P G F T P F R D T-OH	7	Novel peptide
Chemotactic Peptides			
Polybia-CP	I L G T I L G L L K S L-NH_2	16	De Souza et al., 2005
Polybia-CP 2	I L G T I L G K I L-OH	11	Novel peptide
Polybia- CP 3	I L G T I L G T F KS L-NH_2	17	Novel peptide
Protonectin (1–6)	I L G T I L- OH	8	Mendes et al., 2004
Mastoparans			
Polybia-MP I	I D W K K L L D A A K Q I L-NH_2	13	De Souza et al., 2005
Polybia-MP II	I N W L K L G K M V I D A L-NH_2	15A	De Souza et al., 2005
Polybia-MP IV	I D W L K L R V I S V I D L-NH_2	19	Novel peptide
Polybia-MP V	I N W H D I A I K N I D A L-NH_2	15B	Novel peptide
Polybia-MP VI	I D W L K L G K M V M-OH	10	Novel peptide
Unknown function			
unk-1	I P A G W A I V K V–NH_2	9	novel peptide
unk-2	T G D S P D V R–OH	5	novel peptide

sequence to be IPAGWAIVKV-NH2 (Table 1).

Fraction 10 eluted at 32.0 min with a MS^1 spectrum (Fig. S2C in the supplemental information) that revealed the molecular mass to be 1332.80 Da. Using the analytical strategies already mentioned above for the other peptides, the complete sequence of this peptide was assigned as IDWLKIGKMVM-OH. The peptide component from fraction 11 eluted at 34.1 min, and its MS^1 (Fig. S2D in the supplemental information) spectrum indicated that its molecular mass is 1039.72 Da. The use of the protocols mentioned above for solving the isobaric ambiguities and identification of the chemical nature of the C-terminal residue enabled assignment of the complete sequence of this peptide as ILGTILGKIL-OH (Table 1). Fraction 12 was composed of three peptide components at very low concentrations, making it difficult to determine their sequencing. Therefore, they were not analysed.

The sequence assignment was solved by applying the analytical strategies for resolving the isobaric ambiguities and identification of the organic function of the C-terminal residue, resulting in the sequence IDWKKLLDAAKQIL-NH2 (Table 1). Fraction 14 corresponded to a mixture of peptides occurring in tiny amounts and therefore was not studied.

Fraction 15 eluted from 40.0 to 42.0 min and was composed of two different peptide components that partially overlapped one another. Therefore it was treated as fractions 15A and 15B. The MS^1 spectra of the peptides from fractions 15A and 15B (Fig. S3B and S3C in the supplemental information) indicated molecular masses of 1611.98 Da and 1633.95 Da, respectively. The interpretation of MS2 spectra combined with the use of the specific analytical strategies for resolving the isobaric ambiguities, as well the identification of the acidic/amide forms of the C-terminal residue permitted the complete and unambiguous sequencing of both peptides, with Fraction 15A as INWLKLGKMVIDAL-NH2 and Fraction 15B as INWHDIAIKNIDAL-NH2.

Fraction 16 eluted from 41.0 to 41.5 min and consisted of a peptide with a molecular mass of 1238.82 Da (Fig. S4A in the supplemental information). The sequence assigned (after solving the isobaric ambiguities and identification of the chemical form of the C-terminal residue) is ILGTILGLLKSL-NH2 (Table 1). The elution of fraction 17 occurred at 44 min under RP-HPLC (Fig. 1) with a molecular mass of 1260.80 Da (Fig. S4B in the supplemental information); the complete and unambiguous sequence was ILGTILGTFKSL-NH2 (Table 1). The last peptide that was identified eluted at a retention time of 52 min (fraction 19) with a molecular mass of 1680.95 Da (Fig. S4C in the supplemental information); The sequence of this peptide was assigned as IDWLKLRVISVIDL-NH2 (Table 1).

Table 2
Profile of biological activities of the peptides identified in the venom of the social wasp *P. paulista*.

Peptides	Biological Activities*				
	Mast cell degranulation	Hemolysis	PMNLs chemotaxys	Hypernociception	Oedema formation
Thr6-Bradykynin	+	–	–	++	++
RA-Thr^6-Bradykynin	+	–	–	++	++
RA-Thr^6-Bradykynin-DT	+	–	–	++	++
Polybia-CP	+	–	+++	+	+
Polybia-CP 2	+	–	++	+	+
Polybia- CP 3	+	–	++	+	+
Protonectin (1–6)	+	–	++	+	+
Polybia-MP I	+++	+++	+	+++	++
Polybia-MP II	+++	+	+	++	+
Polybia-MP IV	+++	+	+	++	+
Polybia-MP V	++	++	+	++	+
Polybia-MP VI	++	+	+	++	+
unk-1	–	–	+	–	–
unk-2	–	–	+	–	–

(*) All the biological activities were compared against a standard compound as reference for each assay, and expressed as percentages in relation to these standards; (–) means that no activity was detected for the peptide, while (+), (++), and (+++) was established to represent up to 40%, from 41 to 70%, and from 71 to 100% of standard compounds activities.

Table 3
Amino acid sequence alignment of the wasp kinin peptides identified in the venom of the social wasp *P. paulista*, with the sequences of similar peptides from different species of social wasps.

Wasp species	Peptides	Amino acid sequences													
P. paulista	Thr^6-Bradykynin			R	P	P	G	F	T	P	F	R–OH			
	RA-Thr^6-Bradykinin	R	A	R	P	P	G	F	T	P	F	R–OH			
	RA-Thr^6-Bradykinin-DT	R	A	R	P	P	G	F	T	P	F	R	D-OH		T- OH
Polistes rothney	Polisteskinin-R[a]	A	R	R	P	P	G	F	T	P	F	R–OH			
Polistes jadwigae	Polisteskinin-J[a]	R	R	R	P	P	G	F	S	P	F	R–OH			
Pollistes chiensis	Polisteskinin-C[a]	S	K	R	P	P	G	F	S	P	F	R–OH			
Vespa mandarínia	Vespakinin-X[a]		A	R	P	P	G	F	S	P	F	R–OH			
Vespa analis	Vespakinin-A[a]		G	R	P	P	G	F	S	P	F	R	V	I–OH	
	Bradykinin[a]			R	P	P	G	F	S	P	F	R–OH			

[a] Nakajima, 1986.

Table 4
Amino acid sequence alignment of the chemotactic peptides identified in the venom of the social wasp *P. paulista*, with the sequences of similar peptides from different species of social wasps.

Wasp species	Peptides	Amino acid sequences												
P. paulista	Polybia-CP	I	L	G	T	I	L	G	L	L	K	S	L-NH2	
	Polybia-CP 2	I	L	G	T	I	L	G	K	I	L-OH			
	Polybia-CP 3	I	L	G	T	I	L	G	T	F	K	S	L-NH2	
Protonectarina sylveirae	Protonectin(1–6)[a]	I	L	G	T	I	L-OH							
	Protonectin[b]	I	L	G	T	I	L	G	L	L	K	G	L-NH2	
Vepula lewisii	Ves-CP-L[c]	F	L	P	I	I	A	K	L	V	S	G	L-NH2	
Vespa mandarinia	Ves-CP-M[c]	F	L	P	I	L	G	K	L	L	S	G	L-NH2	
Vespa crabro	Crabroline[c]	F	L	P	L	I	L	R	K	I	V	T	A	L-NH2

[a] Mendes et al., 2004.
[b] Dohtsu et al., 1993.
[c] Nakajima, 1986.

To properly identify the fourteen peptides, their sequences were aligned with the sequences of known peptides from wasp venoms (Tables 3–5). The sequence similarities to known peptides were used for grouping and naming the novel peptides, as shown in Table 1. The peptides from fractions 13 (IDWKKLLDAAKQIL-NH2) and 15A (INWLKLGKMVIDAL-NH2) were previously described in *P. paulista* venom as the mastoparans Polybia-MP I, and Polybia-MP II, respectively (De Souza et al., 2005; De Souza and Palma, 2009; Rocha et al., 2010). The peptides from fractions 10, 15B, and 19 were novel mastoparans (Tables 1 and 5) and were named Polybia-MP IV (fraction 19- IDWLKLRVISVIDL-NH2), Polybia-MP V (fraction 15B – INWHDIAIKNIDAL-NH2), and Polybia-MP VI (fraction 10 – IDWLKLGKMVM-NH2) (Table 1).

The peptides from fractions 6A, 6B, and 7 were identified as members of the family of wasp kinins (Tables 1 and 3). The peptide in fraction 6A was identified as Thr^6-Bradykinin (RPPGFTPFR-OH), which was previously observed in the venom of the solitary wasp *Cyphononyx fulvognathus* (Picolo et al., 2010). Meanwhile, the peptides in fractions 6B (RARPPGFTPFR-OH) and 7 (RARPPGFTPFRDT-OH) were traditional for this family of peptides, i.e., RA- Thr^6-Bradykinin, and RA- Thr^6-Bradykinin-DT, respectively. The components of fractions 8, 11, 16, and 17 were identified as chemotactic peptides for PMNLs (Tables 1 and 4). The peptides of fractions 8 (ILGTIL-NH2) and 16 (ILGTILGLLKSL-NH2) were previously reported as chemotactic toxins in social venoms and named Polybia-CP (De Souza et al., 2005) and Protonectin (1–6) (Baptista-Saidemberg et al., 2010), respectively. The components in fractions 11 (ILGTILGKIL-OH) and 17 (ILGTILGTFKSL-NH2) were named Polybia-CP II, and Polybia-CP III, respectively (Tables 1 and 4).

The peptides in fractions 5 (IPAGWAIVKV-NH2) and 9 (TGDSPDVR-OH) did not present any similarity to known peptides from social wasp venoms and thus were designated as unknown

Table 5
Amino acid sequence alignment of the mastoparan peptides identified in the venom of the social wasp *P. paulista*, with the sequences of similar peptides from different species of social wasps.

Wasp species	Peptides	Amino acid sequences													
P. paulista	Polybia-MP I[b]	I	D	W	K	K	L	L	D	A	A	K	Q	I	L-NH2
	Polybia-MP II[b]	I	N	W	L	K	L	G	K	M	V	I	D	A	L-NH2
	Polybia-MP III[a]	I	D	W	L	K	L	G	K	M	V	M	D	V	L-NH2
	Polybia-MP IV	I	D	W	L	K	L	R	V	I	S	V	I	D	L-NH2
	Polybia-MP V	I	N	W	H	D	I	A	I	K	N	I	D	A	L-NH2
	Polybia-MP VI	I	D	W	L	K	L	G	K	M	V	M–OH			
Agelaia pallipes	Agelaia-MP II[c]	I	N	W	K	A	I	L	Q	R	I	K	K	M	L-NH2
	Agelaia-MP I[c]	I	N	W	L	K	L	G	K	A	I	I	D	A	L-NH2
Vespa xanthoptera	Mastoparan X[d]	I	N	W	K	G	I	A	A	M	A	K	K	L	L-NH2
Vespa crabro	Mastoparan C[d]	I	N	W	K	A	L	L	A	V	A	K	K	I	L-NH2
Parapolybia indica	Parapolybia-MP[d]	I	N	L	K	A	L	A	A	L	A	K	K	I	L-NH2

[a] De Souza et al., 2005.
[b] De Souza et al., 2005.
[c] Baptista-Saidemberg et al., 2011.
[d] Nakajima, 1986.

(unk) peptides 1 and 2, respectively. After further study, a novel and appropriate designation will be given to them.

3.1. Bioassays

Because the peptides profiling was performed on-line with an LC-MS and MS^2 system using tiny amounts of *P. paulista* venom, samples were not collected for later use in bioassays. All fourteen peptides that had their sequences completely and unambiguously assigned were synthesized in the solid phase, purified and used for bioassays of mast cell degranulation, haemolysis, chemotaxis of PMNLs, hypernociception, and oedema formation. These biological activities are expressed in comparison to the standard compounds used in each assay; thus, an activity was considered either weak (+), medium (++), or strong (+++) when it represented up to 40%, from 41% to 70%, or $\geq$ 71% of the activity, respectively.

Peptides were organized into four different groups, which in turn were submitted to all the bioassays mentioned above. The group of wasp kinins (Thr^6-Bradykinin, RA- Thr^6-Bradykinin, and RA- Thr^6-Bradykinin-DT) showed weak mast cell degranulation and medium hypernociception activity and oedema formation (Table 2). The group of chemotactic peptides (Polybia-CP, -CP 2, and -CP 3) showed weak mast cell degranulation, weak hypernociception and weak oedema formation but medium to strong chemotaxis for PMNLs (Table 2). The mastoparans (Polybia-MP I, -MP II, -MP IV, -MP V, and −MP VI) showed medium to strong mast cell degranulation, a range of weak to strong haemolytic activity, weak chemotaxis for PMNLs, and a range of weak to strong hypernociception and oedema formation (Table 2). Finally, the group of unknown peptides (unk-1 and -2) presented only weak activity of chemotaxis for PMNLs.

4. Discussion

The venoms of social wasps are used by the worker insects as defensive tools to protect their colonies against attacks by predators (De Souza and Palma, 2009). Approximately 70% of the dry weight of wasp venom is composed of peptide toxins (Palma, 2013). Recently, it was reported that the colonies of *P. paulista* may contain anywhere from 78 to 108 different short linear peptides in the molecular range of 400–3000 Da (Dias et al., 2014). Despite the large number of peptides in this venom, only four have been biochemically characterized: Polybia-MP I, -MP II, and -MP III (De Souza et al., 2005; Mendes and Palma, 2006; Rocha et al., 2010), and Protonectin (1–6) (Baptista-Saidemberg et al., 2011). To increase our knowledge of the biochemical composition of peptide toxins from *P. paulista*, its venom peptidome was profiled to identify some components and their biological activities of these toxins.

Accordingly, *P. paulista* venom was fractionated by RP-HPLC, and analysed by in-line mass spectrometry, constituting an LC-ESI-IT-TOF-MS and MS^n system, as described in Materials and Methods. The chromatographic profile revealed 24 major fractions (Fig. 1), and the analytical strategy applied permitted the unambiguous sequence assignment of fourteen peptides, which were identified and functionally assayed. The peptide components from fractions 6A (Thr^6-Bradykinin), 11 (Polybia-CP 2), 13 (Polybia-MP I), and 15A (Polybia-MP II) were the most abundant peptides in the chromatographic profile. It is important to emphasize that the wasp venom was extracted in the presence of a cocktail of proteinase inhibitors, ensuring that the peptides that the most peptides sequenced were not by-products of uncontrolled proteolysis of the protein fractions during venom extraction. However, the occurrence of a few contaminating peptides is possible, since the venom was obtained by dissecting the venom reservoirs, which may have resulted in the leaking of some material from the venom gland tissue.

The peptides were identified by comparison of their sequences to similar peptide toxins that have previously been characterized and reported in the literature. A group of wasp kinins was identified in *P. paulista* venom as the peptides in fractions 6A, 6B, and 7, which were Thr^6-Bradykinin, RA-Thr^6-Bradykinin, RA-Thr^6-Bradykinin-DT, respectively. The kinins from hymenopteran venoms are structurally similar to the mammalian pain-producing nonapeptide bradykinin (BK) (Jackes and Schachter, 1954; Bhoola et al., 1992; Gobbo et al., 1992; Piek et al., 1983; Yasuhara et al., 1987). Generally the wasp kinins contain an extra sequence attached to the N-terminal end of a bradykinin-like peptide (Piek, 1991) as shown in Table 3. In this study, we describe an uncommon type of wasp kinin because the peptide RA-Thr^6-Bradykinin-DT contains both the dipeptide RA attached to the N-terminal residue of Thr^6-Bradykinin and the dipeptide DT attached to the C-terminal residue of this peptide. Some examples of this type are reported in the literature, including the megascoliakinin (Thr6-BK-Lys-Ala), which was identified in the venom of the solitary wasp *Megascolia flavifrons* (Piek et al., 1987a, 1987b), as well as the wasp kinin A from *Vespa analis* (Nakajima, 1986) (Table 3). There are even some peptides that contain a complete BK sequence within the wasp kinin peptide chain (Nakajima, 1986). Most of the kinin-related peptides in animal venoms have longer peptide chains and their actions are longer-lasting than BK (Piek, 1991). Thr^6-Bradykinin is a neurotoxic component that has already been reported in the venom of solitary wasps *M. flavifrons* and *Colpa interrupta* (Piek et al., 1987a, 1987b), but it is being reported for the first time in the venom of *P. paulista*.

Wasp kinins cause hypertension in mammals, hypotension in chickens, bronchoconstriction in rodents, and the contraction of a series of isolated smooth muscle preparations, and the relaxation of the rat duodenum (Edery et al., 1978). The function of kinin-related peptides in wasp venoms is still not clearly understood. These peptides can activate leukocytes, stimulating the releasing of cytokines, prostaglandins, leukotrienes, reactive oxygen species and the blockage of the cholinergic transmission in the insect central nervous system (CNS) (Mendes and Palma, 2006; Piek, 1991). In this study, three wasp kinin peptides caused weak mast cell degranulation and pain and oedema formation. These observations suggest that wasp kinins may be used as toxins for defence against mammals and/or birds, which are predators of social wasp colonies, causing physical discomfort in the victims of their stings.

The second group of peptides identified in *P. paulista* venom was related to the chemotaxis of PMNLs: fractions 8 (Protonectin (1–6)), 11 (Polybia-CP 2), 16 (Polybia-CP), and 17 (Polybia-CP 3) represent components of chemotactic peptides family. These peptides cause massive recruitment of PMNLs, inducing them to deliver large amounts of oxygen peroxide around the site of wasps stings, resulting in hyperoxidation in the surrounding tissues, followed by subsequent cellular death (Palma, 2013).

The venoms of social wasps generally contain two different types of chemotactic peptides. The first is the classical deca- and dodecapeptides, which contain a single K residue between positions 8 and 10 of the peptide chain and can induce chemotaxis in leukocytes (Baptista-Saidemberg et al., 2010; Mendes et al., 2004). The second type consists of a series of small chemotactic peptides (from tetra-to octapeptides) that act in modulating the chemo attraction of leukocytes. This second type also inhibit catecholamine secretion by non-competitively blocking specific acetylcholine receptors, but without affecting the catecholamine release that is caused in the presence of high concentrations of potassium ions (Monoi et al., 2004). These peptides appear to be natural proteolytic products of serine-proteinases on mastoparans and chemotactic peptides (Xu et al., 2006). Of the peptides identified in the present study, Polybia-CP, Polybia-CP 2, and Polybia-CP 3 are representative of the first type of chemotactic components, whereas Protonectin

(1–6) is a representative of the second type. In addition to the chemotaxis of PMNLs, these peptides also contained mast cell degranulation activity, hypernociception, and oedema formation, which corroborate the multifunctional nature of the toxins.

Table 4 shows an alignment of the four chemotactic peptides that were observed in *P. paulista* venom, in comparison to similar peptides isolated from other wasp species. The peptide Polybia-CP was previously reported in the same venom (Mendes et al., 2004), whereas the Polybia-CP 2 and Polybia-CP 3 peptides are novel toxins. Protonectin was previously reported in the venom of the social wasp *Protonectarina sylveirae* (Dohtsu et al., 1993), *Agelaia p. pallipes* (Mendes et al., 2004), and *Polistes rothneyi iwatai* (Polistes-Protonectin) (Murata et al., 2009). It is notable that protonectin (1–6) is the only peptide of this group with the C-terminal residue in its free acid form; the other peptides contain amides at this position. A comparison of the sequence of Polybia-CP and Protonectin reveals that the only difference is the presence of an S at the 11th position of Polybia-CP, whereas this position is G in Protonectin (Table 4). Table 4 also reveals an interesting structural feature of this group of toxins, in that the chemotactic peptide isolated from the venoms of social wasps that are endemic to the Neotropics (*P. paulista* and *P. sylveirae*) contain a highly conserved sequence ILGTIL; this motif may be considered a molecular signature of this group. Meanwhile, the chemotactic peptides isolated from wasps species that are endemic to the northern hemisphere (*Vepula lewisii*, *Vespa mandarinia*, and *Vespa crabro*) have the sequence FLP at the N-terminal region as their molecular signature.

The third group of peptides identified in *P. paulista* venom are represented by the mastoparans, which were in fraction 13 (Polybia-MP I), fraction 15A (Polybia-MP II), fraction 19 (Polybia-MP IV), fraction 15B (Polybia-MP V), and fraction 10 (Polybia-MP VI (Table 1). These peptides constitute the most numerous and abundant toxins in *P. paulista* venom. Mastoparans cause potent mast cell degranulation and also may cause haemolysis, PMNLs chemotaxis, hypernociception, and oedema formation, which were confirmed by the bioassays summarized in Table 2. Typically, most mastoparans are tetradecapeptides containing from two to four K residues at positions 4/5 and/or 11/12 and an amide C-terminal residue (Dos Santos Cabrera et al., 2009). The positioning of K residues at these sites can contribute to flanking and maintaining stable helical chains as well as a more homogeneous hydrophobic surface for these molecules in an amphipathic structure. Such a surface could contribute to the lytic activity of these peptides (De Souza and Palma, 2008; De Souza et al., 2011). This structural feature also gives the mastoparans antimicrobial activity and the potential to cause cytolytic action in animal cells (Dos Santos Cabrera et al., 2008).

It has been reported that mastoparans play important roles in allergy and inflammation because they activate exocytosis and granule fusion with the plasma membrane, with the consequent releasing of histamine, proteases, lipid mediators and cytokines (Puri and Roche, 2008). Mastoparans act on mast cells through two mechanisms: binding to specific sites of the G_{α}-subunit of heterotrimeric G-protein receptors, stimulating the guanine nucleotide exchange and leading to a cascade of events, including an increase in the intracellular levels of Ca^{+2} ions, which in turn activates a downstream cascade that results in exocytosis; and binding to specific proteins in endosomal membranes, which activates a Ca^{+2}-independent FcεRI-mediated exocytosis pathway (Chahdi et al., 2004; Miles et al., 2004; Santos et al., 2012). Mastoparan peptides are multifunctional toxins that are involved at different levels to produce pain, oedema and inflammation.

Table 5 shows the alignment of the mastoparans that were identified in this study compared to those previously identified in the venom of the same wasp species as well as in other social wasp venoms. The peptides Polybia-MP I and Polybia-MP II were also identified in this study but were already reported in a previous investigation by our group (De Souza and Palma, 2009). The peptide Polybia-MP III was previously identified in *P. paulista* venom (De Souza et al., 2005) but was not observed in the present investigation. The mastoparans Polybia-MP IV, Polybia-MP V, and Polybia-MP VI constitute novel peptide toxins in this venom. It is important to emphasize that the only mastoparan peptide that has been reported to contain a C-terminal residue in free acidic form is the Polybia-MP VI, which in turn, is poorly active in relation to all the activities assayed in the present study. These results support the expected structure/activity relationship for a mastoparan peptide in this condition (Saidemberg et al., 2011; Sforza et al., 2004; Silva et al., 2014). Most mastoparans already reported in the literature contain a molecular signature in the motif IDW or INW in the N-terminal region of the peptide chain as is shown in Table 5.

Two novel peptides were identified in fractions 5 (unk-2) and 9 (unk-1), which contained weak mast cell degranulation activity, but their sequences were not conserved in relation to any known family of Hymenopteran venom peptides. It may be considered that these peptides are contaminants leaked from the venom glands during reservoirs dissection, as already mentioned above. Anyway, their functional characterization will require further investigation in the future.

The mass chromatogram of the peptide-rich fraction (Fig. 1) was used to determine the individual concentration of each peptide; thus, it was observed that the most concentrated peptide components in *P. paulista* venom were those from fractions 6A (Thr6-Bradykinin), 10 (Polybia MP-VI), 11 (Polybia CP-2), and 13 (Polybia MP-I), which occurred at levels of 460, 182, 360, and 550 ng/venom gland, respectively. Meanwhile, the less abundant component was that from fraction 17 (Polybia CP-3) which occurred at level of 16 ng/venom gland. Thus, most prominent peptides from *P. paulista* venom are the Thr6-Bradykinin, mastoparans, and chemotactic peptides.

5. Conclusion

P. paulista is a very aggressive social wasp that is endemic to Southeast Brazil, where it causes many incidents of severe envenoming each year; the venom of this wasp was submitted to mass spectrometric analysis to profile its peptidome. Fourteen major peptides were detected and sequenced. Five of them have already been reported and characterized in previous publications. Nine of them are novel toxins in *P. paulista* venom. The novel peptides correspond to two wasp kinins, two chemotactic components, three mastoparans, and two peptides of unknown function. The novel peptides appear to act synergistically with the previously known toxins and constitute three well-known families of peptide toxins (wasp kinins, chemotactic peptides, and mastoparans). The venom is used to promote mnemonic actions in sting victims through the uncomfortable inflammatory response to the toxin composition, and specifically the peptides (De Souza and Palma, 2009). These peptides are multifunctional toxins that cause a series of different actions (pain, oedema formation, haemolysis, chemotaxis of PMNLs, and mast cell degranulation) in wasp sting victims to generate a strong inflammatory response and cause physical discomfort in the victims. Understanding the complex matrix of peptides composing the venom of *P. paulista* will contribute to a better understanding the complex mechanism of pathogenesis caused by this venom.

Ethical statement

The authors declare that all experiments were performed in accordance with the guidelines for the ethical use of conscious

animals in pain research, published by the National Academy of Sciences. The procedures were approved by the Institutional Animal Care Committee at São Paulo State University, UNESP campus Rio Claro, SP (CEUA-IB-UNESP-CRC, Protocol nº 1984). Efforts were made to minimize the number of animals used and their suffering.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.08.013.

Transparency document

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References

Baptista-Saidemberg, N.B., Saidemberg, D.M., De Souza, B.M., César-Tognoli, L.M.M., Ferreira, V.M.R., Mendes, M.A., Dos Santos Cabrera, M.P., Ruggiero Neto, J.R., Palma, M.S., 2010. Protonectin (1–6): a novel chemotactic peptide from the venom of the social wasp *Agelaia pallipes pallipes*. Toxicon 56, 880–889.

Baptista-Saidemberg, N.B., Saidemberg, D.M., Palma, M.S., 2011. Profiling the peptidome of the venom from the social wasp *Agelaia pallipes pallipes*. Profiling the peptidome of the venom from the social wasp *Agelaia pallipes Pallipes*. J. Proteomics 74, 2123–2137.

Bhoola, K.D., Figueroa, C.D., Worthy, K., 1992. Bioregulation of kinins: kallikreins, kininogens and kininases. Pharmacol. Rev. 44, 1–80.

Biass, D., Dutertre, S., Gerbault, A., Menou, J.L., Offord, R., Favreau, P., Stocklin, R., 2009. Comparative proteomic study of the venom of the piscivorous cone snail *Conus consors*. J. Proteomics 72, 210–218.

Brigatte, P., Cury, Y., De Souza, B.M., Baptista-Saidemberg, N.B., Saidemberg, D.M., Gutierrez, V.P., Palma, M.S., 2011. Hyperalgesic and edematogenic effects of peptides isolated from the venoms of honeybee (*Apis mellifera*) and Neotropical social wasps (*Polybia paulista* and *Protonectarina sylveirae*). Amino Acids 40, 101–111.

Calvete, J.J., Juarez, P., Sanz, L., 2007. Snake venomics: strategy and applications. J. Mass Spectrom. 42, 1405–1414.

Chahdi, A., Sorokin, A., Dunn, M.J., Landry, Y., 2004. The Rac/Cdc42 guanine nucleotide exchange factor β1Pix enhances mastoparan-activated G_i-dependent pathway in mast cells. Biochem. Biophys. Res. Commun. 317, 384–389.

Conlon, J.M., Al-Ghafari, N., Coquet, L., Leprince, J., Jouenne, T., Vaudry, H., Davidson, C., 2006. Evidence from peptidomic analysis of skin secretions that the red-legged frogs, *Rana aurora draytonii* and *Rana aurora aurora*, are distinct species. Peptides 27, 1305–1312.

Davis, J., Jones, A., Lewis, R.J., 2009. Remarkable inter- and intra-species complexity of conotoxins revealed by LC/MS. Peptides 30, 1222–1227.

De Graaf, D.C., Alerts, M., Danneels, E., Devreese, B., 2009. Bee, wasp and ant venomics pave the way for a component resolved diagnosis of sting allergy. J. Proteomics 72, 145–154.

De Souza, B.M., Mendes, M.A., Santos, L.D., Marques, M.R., César, L.M.M., Almeida, R.N.A., Pagnocca, F.C., Konno, K., Palma, M.S., 2005. Structural and functional characterization of two novel peptide toxins isolated from the venom of the social wasp *Polybia paulista*. Peptides 26, 2157–2164.

De Souza, B.M., Palma, M.S., 2008. Monitoring the positioning of short polycationic peptides in model lipid bilayers by combining hydrogen/deuterium exchange and electrospray ionization mass spectrometry. Biochim. Biophys. Acta Biomembr. 1778, 2797–2805.

De Souza, B.M., Palma, M.S., 2009. Peptides from Hymenoptera venoms: biochemistry, pharmacology and potential applications in health and biotechnology. In: De Lima, M.E., Pimenta, A.M.C., Martin-Euclaire, M.F., Zingali, R.B., Organizers (Eds.), Animal Toxins: the State of Art. Perspectives on Health and Biotechnology; Belo Horizonte. Editora UFMG, Brazil, pp. 273–297.

De Souza, B.M., Dos Santos Cabrera, M.P., Ruggiero-Neto, J., Palma, M.S., 2011. Investigating the effect of different positioning of lysine residues along the peptide chain of mastoparans for their secondary structures and biological activities. Amino Acids 40, 77–90.

Dias, N.B., de Souza, B.M., Gomes, P.C., Palma, M.S., 2014. Peptide diversity in the venom of the social wasp *Polybia paulista* (Hymenoptera): a comparison of the intra- and inter-colony compositions. Peptides 51, 122–130.

Dohtsu, K., Okumura, K., Palma, M.S., Nakajima, T., 1993. Isolation and sequence analysis of peptides from the venom of *Protonectarina sylveirae* (Hymenoptera – Vespidae). Nat. Toxin 1, 271–276.

Dos Santos Cabrera, M.P., Costa, S.T., De Souza, B.M., Palma, M.S., Ruggiero, J.R., Ruggiero-Neto, J., 2008. Selectivity in the mechanism of action of antimicrobial mastoparan peptide Polybia-MP1. Eur. Biophys. J. 37, 879–891.

Dos Santos Cabrera, M.P., Arcisio-Miranda, M., da Costa, L.C., De Souza, B.M., Broggio-Costa, S.T., Palma, M.S., Ruggiero-Neto, J., Procopio, J., 2009. Interactions of mast cell degranulating peptides with model membranes: a comparative biophysical study. Arch. Biochem. Biophys. 486, 1–11.

Dyason, K., Brandt, W., Prendini, L., Verdonck, F., Tytgat, J., du Plessis, J., Muller, G., van der Walt, J., 2002. Determination of species specific components in the venom of *Parabuthus scorpions* from southern Africa using matrix-assisted laser desorption time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 16, 768–773.

Edery, H., Ishay, J., Gitter, S., Joshua, H., 1978. Venom of Vespidae. In: Bettini, S. (Ed.), Arthropod Venoms. Springer, Berlin, pp. 691–771.

Escoubas, P., 1998. Multidimensional peptide fingerprinting by high performance liquid chromatography, capillary zone electrophoresis and matrix assisted laser desorption/ionization time-of-flight mass spectrometry for the identification of tarantula venom samples. Rapid Commun. Mass Spectrom. 12, 1075–1084.

Escoubas, P., Sollod, B.L., King, G.F., 2008a. Venom landscapes: mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. Toxicon 47, 650–663.

Escoubas, P., Quinton, L., Nicholson, G.M., 2008b. Venomics: unravelling the complexity of animal venoms with mass spectrometry. J. Mass Spectrom. 43, 279–295.

Gobbo, M., Biondi, L., Filira, F., Scolara, B., Rocchi, R., Piek, T., 1992. Synthesis and biological activity of the non- and digalactosyl- vespulakinin analogues. Int. J. Pept. Protein Res. 40, 54–61.

Jacques, R., Schachter, M., 1954. The presence of histamine, 5- hydroxytryptamin and a potent, slow-contracting substance in wasp venom. Br. J. Pharmacol. 9, 53–58.

Jia, C., Qi, W., He, Z., Qiao, B., 2006. Sequencing peptides by electrospray ion-trap mass spectrometry: a useful tool in synthesis of Axinastatin 3. Cent. Eur. J. Chem. 4, 620–631.

Liang, S., 2008. Proteome and peptidome profiling of spider venoms. Expert Rev. Proteomics 5, 1075–1084.

Mandal, A.K., Ramasamy, M.R., Sabareesh, V., Openshaw, M.E., Krishnan, K.S., Balaram, P., 2007. Sequencing of T-superfamily conotoxins from Conus virgo: pyroglutamic acid identification and disulfide arrangement by MALDI mass spectrometry. J. Am. Soc. Mass Spectrom. 18, 1396–1404.

Mendes, M.A., De Souza, B.M., Marques, M.R., Palma, M.S., 2004. Structural and biological characterization of two novel peptides from the venom of the neotropical social wasp *Agelaia pallipes pallipes*. Toxicon 44, 67–74.

Mendes, M.A., Palma, M.S., 2006. Two new bradykinin-related peptides from the venom of the social wasp Protopolybia exigua (Saussure). Peptides 27, 2632–2639.

Miles, G.P., Marcus, A.M.A., Jones, A.M., Ellis, B.E., 2004. Mastoparan rapidly activates plant MAP kinase signaling independent of heterotrimeric G proteins. Plant Physiol. 134, 1332–1336.

Monoi, N., Usui, M., Takahashi, S., Ito, H., Kamakura, K., Kasai, H., 2004. Tetrapeptides on N- and C-teminal regions of mastoparan inhibit catecholamine release from chromaffin cells by blocking nicotinin acetylcholine receptor. Cell. Mol. Neurobiol. 24, 37–50.

Murata, K., Shinada, T., Ohfune, Y., Hisada, M., Yasuda, A., Naoki, H., Nakajima, T., 2009. Novel mastoparan and protonectin analogs isolated from a solitary wasp, *Orancistrocerus drewseni drewseni*. Amino Acids 37, 389–394.

Nakajima, T., 1984. Biochemistry of vespid venoms. In: Tu, A.T. (Ed.), Handbook of Natural Toxins. Marcel Dekker, pp. 309–327.

Nakajima, T., 1986. Pharmacological biochemistry of vespid venoms. In: Piek, T. (Ed.), Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects. Academic Press Inc. Ltd, London, pp. 309–327.

National Academy of Sciences, 1996. Guide for the Care and Use of Laboratory Animals, seventh ed. National Research Council, Institute for Laboratory Animal Research, NAS, Washington, D.C. http://www.nap.edu/catalog/5140.html

Newton, K.A., Clench, M.R., Deshmukh, R., Jeyaseelan, K., Strong, P.N., 2007. Mass fingerprinting of toxic fractions from the venom of the Indian red scorpion, *Mesobuthus tamulus*: biotope-specific variation in the expression of venom peptides. Rapid Commun. Mass Spectrom. 21, 3467–3476.

Palma, M.S., 2011. Peptides as toxins/defensins. Amino Acids 40, 1–4.

Palma, M.S., 2013. Hymenoptera venom peptides (Chapter 58) p. 416 – 422. In: Kastin, A. (Ed.), Handbook of Biologically Active Peptides, second ed. Academic Press, San Diego, USA, p. 1942.

Picolo, G., Hisada, M., Moura, A.B., Machado, M.F.M., Sciani, J.M., Conceição, I., Melo, R.L., Oliveira, V., Lima-Landman, M.T., Cury, Y., Konno, K.,1, Hayashi, M.A.F., 2010. Bradykinin-related peptides in the venom of the solitary wasp *Cyphononyx fulvognathus*. Biochem. Pharmacol. 79, 478–486.

Piek, T., Buitenhuis, A., Simonthomas, R.T., Ufkes, J.G.R., Mantel, P., 1983. Smooth muscle contracting compounds in the venom of *Megascolia flavifrons* (Hym: Scoliidae) with notes on the stinging behaviour. Comp. Biochem. Physiol. 75C, 145–152.

Piek, T., Hue, B., Pelhate, M., Mony, L., 1987a. The venom of the wasp *Campsomeris sexmaculata* (F.) block synaptic transmission in insect CNS. Comp. Biochem. Physiol. 87C, 283–286.

Piek, T., Hue, B., Mony, L., Nakajima, T., Pelhate, M., Yasuhara, T., 1987b. Block of synaptic transmission in insect CNS by toxins from the venom of the wasp *Megascolia flavifrons* (FAB). Comp. Biochem. Physiol. 87C, 287–295.

Piek, T., 1991. Neurotoxic kinins from wasp and ant venoms. Toxicon 29, 139–149.

Puri, N., Roche, P.A., 2008. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. Proc. Nat. Acad. Sci. 105, 2580–2585.

Rocha, T., Barros, L.S.B., Fontana, K., De Souza, B.M., Palma, M.S., Cruz-Höfling, M.A., 2010. Inflammation and apoptosis induced by mastoparan Polybia-MPII on skeletal muscle. Toxicon 55, 1213–1221.

Saidemberg, D.M., Baptista-Saidemberg, N.B., Palma, M.S., 2011. Chemometric analysis of Hymenoptera toxins and defensins: a model for predicting the biological activity of novel peptides from venoms and hemolymph. Peptides 32, 1924–1933.

Santos, L.D., Pinto, J.R.A.S., Menegasso, A.R.S., Saidemberg, D.M., Garcia, A.M.C., Palma, M.S., 2012. Proteomic profiling of the molecular targets of interactions of the mastoparan peptide Protopolybia MP-III at the level of endosomal membranes from rat mast cells. Proteomics 12, 2682–2693.

Sforza, M.L., Oyama Jr., S., Canduri, F., Lorenzi, C.C.B., Pertinhez, T., Konno, K., De Souza, B.M., Palma, M.S., Ruggiero-Neto, J., Azevedo Jr., W.F., Spisni, A., 2004. How C-terminal carboxyamidation alters the biological activity of peptides from the venom of the Eumenine solitary wasp. Biochemistry 43, 5608–5617.

Silva, A.V., Souza, B.M., Santos Cabrera, M.P., Dias, N.B., Gomes, P.C., Ruggiero-Neto, J., Stabeli, R.G., Palma, M.S., 2014. The effects of the C-terminal amidation of mastoparans on their biological actions and interactions with membrane-mimetic systems. Biochim. Biophys. Acta – Biomembr. 1838, 2357–2368.

Xu, X., Li, J., Lu, Q., Yang, H., Zhang, Y., Lai, R., 2006. Two families of antimicrobial peptides from wasp (*Vespa magnifica*) venom. Toxicon 47, 249–253.

Yasuhara, T., Mantel, P., Nakajima, T., Piek, T., 1987. Two kinins isolated from an extract of the venom reservoirs of the solitary wasp *Megascolia flavifrons*. Toxicon 25, 527–535.